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<b>(21) International Application Number:</b> PCT/DK89/00059 <b>(22) International Filing Date:</b> 17 March 1989 (17.03.89) <b>(31) Priority Application Numbers:</b> 1453/88 737/89 <b>(32) Priority Dates:</b> 17 March 1988 (17.03.88) 17 February 1989 (17.02.89) <b>(33) Priority Country:</b> DK <b>(71) Applicant (for all designated States except US):</b> NOR- DISK GENTOFTE A/S [DK/DK]; Niels Steensens- vej 1, DK-2820 Gentofte (DK). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only) :</b> FLODGAARD, Hans [DK/DK]; Melvillevej 6, DK-2900 Hellerup (DK). ØSTERGAARD, Erik [DK/DK]; Børglumvej 19B 1.tv., DK-2720 Vanløse (DK). THOMSEN, Jo- hannes [DK/DK]; Fasanvænget 506, DK-2980 Kok- kedal (DK). BAYNE, Stephen [GB/DK]; Pilevej 16, Tune, DK-4000 Roskilde (DK).		<b>(74) Agent:</b> HOFMAN-BANG & BOUTARD A/S; Adel- gade 15, DK-1304 Copenhagen K (DK).  <b>(81) Designated States:</b> AT, AT (European patent), AU, BB, BE (European patent), BG, BJ (OAPI patent), BR, CF (OAPI patent), CG (OAPI patent), CH, CH (Eu- ropean patent), CM (OAPI patent), DE, DE (Euro- pean patent), DK, FI, FR (European patent), GA (OAPI patent), GB, GB (European patent), HU, IT (European patent), JP, KP, KR, LK, LU, LU (Euro- pean patent), MC, MG, ML (OAPI patent), MR (OA- PI patent), MW, NL, NL (European patent), NO, RO, SD, SE, SE (European patent), SN (OAPI pa- tent), SU, TD (OAPI patent), TG (OAPI patent), US.  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> HEPARIN-BINDING PROTEINS, DNA CUDING FOR THEM, PROCESSES FOR PRODUCING THEM AS WELL AS THERAPEUTIC PREPARATIONS CONTAINING THEM  <b>(57) Abstract</b>  A heparin-binding protein (HBP) which has, in glycosylated state, an apparent molecular weight of about 28 kDa, determined by SDS-PAGE under reducing conditions, and exhibits angiogenic properties in vivo.		

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Heparin-binding proteins, DNA coding for them, processes for producing them as well as therapeutic preparations containing them

The present invention concerns heretofore unknown heparin-binding proteins or equivalent modifications  
5 hereof which release angiogenesis and improved granulation tissue formation in wounds in animal models. The invention also relates to processes for producing the proteins and pharmaceutical preparations containing the proteins suitable for stimulation of tissue repair  
10 in man, in particular for topical application to external wounds. The proteins are furthermore characterized by in the normal condition being glycoproteins.

15 Normal tissue repair follows an orderly sequence of cellular and biochemical events, which is initiated by injury and results in new tissue formation.

Resting fibroblasts at the wound edge divide, migrate  
20 towards the avascular wound space, and produce collagen. New capillaries bud from preexisting venules and capillaries and migrate towards the wound edge. These processes continue until the edge of the healing wound fuses, filling the wound space with a vascularized  
25 collagen fibroblast mesh (granulation tissue). Finally, epithel cells divide and cover the granulation tissue and the repair process is finished.

The blood platelets are the first cellular element of  
30 importance for the healing of an acute wound within the first 24 hours. Then the healing processes are taken over by neutrophilic granulocytes followed by macrohages and lymphocytes, which can all be seen to migrate into the wound in an orderly sequence during the next 2 to 3  
35 days after the tissue damage. It is these inflammatory cells which eventually ensure correct healing via carefully adapted release of paracrinic growth factors.

In recent years some understanding has accumulated concerning the mechanism for the action for these growth factors, which are called Platelet derived growth factor (PDGF), Transforming growth factor alpha (TGF $\alpha$ ), and Transforming growth factor  $\beta$  (TGF $\beta$ ) in the wound healing process. PDGF is initially released from the  $\alpha$ -granules of the blood platelets when the platelets adhere to the edges of the fresh wound and have strong chemotaxis for fibroblasts (Grotendorst, G.R. et al. (1981). Proc. Natl. Acad. Sci. USA. 78: 3669-3672) in addition to being mitogenic to the same cells in the presence of either TGF $\alpha$  or epidermal growth factor (EGF) (Deuel, T.F. et al. (1985) Cancer Surv. 4: 633-653.

PDGF activates fibroblasts to release collagenase (Bauer, E.A. et al. (1985) Proc. Natl. Acad. Sci. USA, 82: 4132-4136) and thus contributes to remodelling the matrix, an essential element in the wound healing.

TGF $\beta$  is found in relatively high concentrations in the blood platelets and is also shown to be released from  $\alpha$  granules during the clot formation. This growth factor plays an important part in the matrix formation in wounds and has also been found to have a regulatory influence on a variety of other growth factors, such as PDGF, EGF, and TGF $\alpha$ .

TGF $\beta$  exhibits strong chemotactic activity for monocytes. Thus the growth factors initially released from platelets immediately after wounding also play an important role by stimulating migration of inflammatory cells to the wound. TGF $\beta$  may recruit monocytes from the circulation and subsequently activate them to the secretory phenotype (Wiseman, D.M. et al. (1988) Biochemical and Biophysical Research Communications 157, 793-800).

Once the monocytes have obtained this phenotype and are now better named macrophages, they can be shown to secrete the same factors found in platelets plus several others of great importance to the repair process.

Thus monocytes/macrophages excrete growth regulatory factors, such as Platelet derived growth factor (PDGF), Transforming growth factor beta (TGF $\beta$ ), Transforming growth factor alpha (TGF $\alpha$ ), Basic fibroblast growth factor (bFGF). Insulin-like growth factor one (IGF-1) Bombesin, Granulocyte-stimulating factor (GSF), Granulocyte-macrophage colony stimulating factor (GM-CSF), Monocyte stimulating factor (M-CSF) and Interleukin-1 (IL-1). The secretory products also include proteases, complement proteins, monocyte-derived neutrophil-activating factor, arachidonates and Tumor necrosis factor alpha (TNF $\alpha$ ). (Rom, W.N. et al. (1988) J. Clin. Invest. 82, 1685-1693), Rappolee D.A. et al (1988) Science 241, 708-712), (for review see Unanue, E.R. et al (1987). Science 236, 551-557).

All these macrophage derived paracrinic growth factors participate in the healing process, but many details concerning the mechanism and the complex interaction among these factors are still poorly understood.

During the healing process it is of decisive importance that the growing tissue is sufficiently provided with oxygen and nutrients. This is secured by the complicated process known as angiogenesis which leads to formation of new blood vessels in situ. This process involves the orderly migration, proliferation and differentiation of vascular cells, (Folkman, M. et al. (1987). Science 235, 442). The initiation of angiogenesis by direct stimulation of endothelial cell proliferation is the presumed responsibility of two polypeptide mitogens: The

class I heparin-binding growth factor (HBGF-1), also known as acidic fibroblast growth factor, and class II heparin-binding growth factor (HBGF-II) or basic fibroblast growth factor (bFGF).

5

(Thomas, K.A. (1985). Proc. Natl. Acad. Sci. 82, 6409), Esch, F. (1985) *ibid*: 6507). These factors are not found in platelets, but basic FGF is secreted from activated monocytes/macrophages as mentioned above and have been shown to induce angiogenesis in animal models *in vivo*.

10

It is therefore clear that the initial "burst" of platelet release in connection with wounding does not involve direct angiogenic factors. However, platelet extracts are shown to be angiogenic in in vivo experiments and this can be shown to be a result of monocyte activation which in turn leads to secretion of the relevant factors mentioned above. Several attempts to isolate and characterize a non-mitogenic angiogenic factor in platelets have been done, but the nature of this factor is not disclosed in the arts (Knighton, D.R. et al., 1986, Ann. Surg. 204, 323-331).

15

20

A necessary requisite for angiogenesis to occur is the presence of heparin in the wound area, and it has been shown that removal of heparin with e.g. protamine completely abolishes angiogenesis.

25

Therefore any factor having the capability of recruiting monocytes from the circulation to the wounded area and subsequently activate them in addition to having heparin-binding properties, must be of extreme importance for angiogenesis and the whole repair process as well.

30

35

The present invention provides heretofore unknown proteins (the human and porcine types are hereinafter

referred to as hHBP and pHBP) which are uniquely suited to stimulate angiogenesis and tissue repair for the following reasons:

- 5 a) The proteins are released from platelets when these cells are activated such as it occurs in the damaged tissue.
- b) The proteins bind to heparin.
- 10 c) The proteins are chemotactic for monocytes.
- d) The proteins activate monocytes morphologically towards the secretory phenotype.
- 15 e) The proteins activate monocytes in culture to excrete mitogens for fibroblasts in culture.
- f) Application of the proteins gives rise to
- 20 angiogenesis in the hen egg chorio-allantoic membrane model.
- g) The proteins increase epithelialization rate when applied to wound-chambers in experimental models in
- 25 rats judged from macroscopic and histological examinations.
- h) The proteins increase granulation tissue formation in the same wound-chamber model judged from
- 30 macroscopic and histological examinations.
- i) The proteins increase blood vessel formation in the same rat wound-chamber model judged from macroscopic
- 35 examinations.

Two specific examples of hitherto unknown proteins are derived from porcine and human platelets the amino acid

sequence of the porcine type has been fully elucidated and covered by claim 4. The human type is strongly homologous to the porcine type as evident by comparing the amino acid sequence of the porcine type as disclosed in claim 4 with the amino acid sequence for the human type as disclosed in claim 10.

An important feature in relation to tissue repair is the strong heparin-binding properties of the proteins. Shortly after tissue damage it can be observed that connective tissue mast cells among several components of importance for the inflammation also release large amounts of heparin. (Qureshi, R. et al. (1988). The Journal of Immunology 141, 2090-2096).

The released heparin is known to bind to collagen and once this has been established, the heparin-binding proteins (HBP) described in this invention thereby become immobilized. This can form a fixed gradient and as Gustafson et al. have suggested on theoretical grounds and Carter has shown experimentally, cells tend to move up a gradient of increasing substrate adhesion. Carter has suggested that this phenomenon should be called "Haptotaxis" (Greek: haptain, to fasten; taxis, arrangement). On this basis, the cell migration involved in morphogenesis, inflammation, wound healing, tumour invasion and indeed all tissue cells movements, are considered to be the result of haptotactic responses by the cells involved (Gustafson, T. et al. (1963). Intern. Rev. Cytol, 15, 139), Carter, S.B. (1965), Nature 208, 1183-1187).

On this basis the heparin-binding proteins said herein are uniquely suited to recruit monocytes from the circulation into the damaged tissue area. The subsequent activation to secretory phenotype takes place in situ i.e. the complete cocktail of monocyte/macrophage



derived cytokins releases in the neighbourhood of the damaged cells in the wound and facilitate healing.

The heparin-binding proteins herein are foreseen especially to be suitable to stimulate healing of chronic wounds in man. It is generally believed that the most important pathogenic factor for the chronic leg ulcers and decubitus in elderly patients is the lack of neovascularization (angiogenesis).

On the basis of the properties mentioned for the heparin-binding proteins external application of the proteins (preferably the human type) to chronic wounds are foreseen to accelerate healing. Ablation of macrophages slows the wound-healing response (Leibovich, S. J. et al. (1975). Am. J. Pathol. 78, 71). In the human clinic such depletion of macrophages accompanies several illnesses, and it is also often a result of therapy. Thus, severe leucocytopenia is observed in cancer patients treated with chemotherapy or radiation therapy. Slow healing after surgical treatment and occurrence of chronic ulcers is often seen in such patients. The special properties shown by the heparin-binding proteins may be of therapeutically benefits in such patient groups.

HBP may also be used in the therapy of severe burns. The lack of neovascularization results in poor healing and the damaged tissue is susceptible to infections. Therefore, a component having monocyte activating properties may be of great advantage. Activated monocytes or "macrophages" act as scavengers, and by phagocytosis they remove damaged tissue debris, a most essential function in connection with burns.

More recently, the growth regulatory role of macrophages with respect to tumor growth has received considerable

attention.

High concentrations of activated macrophages are  
cystostatic to neoplastic cells, and this effect is  
5 directed selectively against neoplastic target cells.  
The putative secretory product from macrophages in this  
context is believed to be  $\text{TNF}\alpha$  (Diegelmann, R.F. et al.  
1981, Plastic and Reconstructive Surgery 1968, 107-113)

10 The heparin-binding proteins said herein may have  
therapeutic possibilities in tumor therapy. HBP injected  
to solid tumors can recruit circulating monocytes to the  
tumor area and by subsequent activation mediate  
cytotoxic effect.

15  
Pharmaceutical compositions for use in the present  
invention in the clinic as suggested above include  
incorporation of human HBP into creams, ointments, gels,  
foams, dressing materials, patches, pads, artificial  
20 skins, aqueous vehicles for soaking gauze dressing, dry  
swellable powders or suture coatings.

The formulations which may be used to entrap HBP are a  
freeze-dried pad or a hydrocolloid occlusive dressing.  
25 It is preferred that a medical dressing which provides  
controlled release of HBP is used.

The gel which may be used consists of an aqueous basis  
which is made highly viscous by adding water-soluble  
30 etherified cellulose derivatives such as alkyl  
cellulose, hydroxyalkyl cellulose and alkylhydroxyalkyl  
celluloses, e.g. methyl cellulose, hydroxyethyl  
cellulose, carboxymethyl cellulose, hydroxypropylmethyl  
cellulose and hydroxypropyl cellulose. The hydroxyalkyl  
35 cellulose derivatives, such as hydroxypropyl cellulose,  
hydroxyethyl cellulose and hydroxypropylmethyl cellulose  
are preferred. Usually HBP is dissolved in the aqueous

phase before the gelling agent is added.

5 A freeze-dried pad may consist of a hydroxycolloid with a coherent fibrous structure formed by lyophilization of a gel. The hydrocolloid may be a water-soluble etherified cellulose derivative as mentioned above. Usually HBP is entrapped in the pads prior to freeze-drying.

10 A medical dressing may be an adhesive occlusive bandage having HBP incorporated in it. The dressing comprises a sealing material, a tackifier as a continuous phase, and a discontinuous phase dispersed in the continuous phase which comprises one or more water-soluble or water-  
15 swellable compounds, such as etherified cellulose derivatives mentioned above. The ability of the discontinuous phase to swell in water gives the possibility of gradual release of previous physically entrapped HBP. HBP may also be administered in liquid  
20 formulations by subcutaneous, intermuscular or intravenous injections. Furthermore, the administration of HBP may also occur by nasal, buccal, rectal or intraperitoneal routes.

25 According to the invention, HBP can be produced from blood platelets, obtained from porcine or human blood. More particularly, the protein is produced by fractionation of a blood platelet extract. Column chromatography using heparin-Sepharose is expedient for  
30 this purpose. Such a chromatographic method comprising gradient elution with NaCl from 0.5 M up to 3 M of a column through which an extract of the blood platelets has been poured first, results in elution of two peaks. The first peak around 1.2 M NaCl can be measured at 280  
35 nm as a large protein peak and is a platelet factor (PF<sub>4</sub>) known per se. Around 1.8 M NaCl, the protein amount is below the detection limit in the system used,

but the fractions in this area have angiogenic activity. The active fractions are purified additionally by microbore reverse phase HPLC on C<sub>4</sub> column, and at 214 nm a completely pure protein peak can be detected, and this  
5 protein is identical with the present HBP's of either porcine or human type depending on the kind of platelets used.

The HBP's may also be produced by recombinant technique.  
10 Bacteria, yeasts, fungi or mammalian cell lines may be used as hosts for the production of HBP. By transformation of the host cell with a suitable vector containing the necessary transcription- and translation signals as well as the DNA-sequence encoding HBP,  
15 production of HBP can be achieved.

A choice can be made as to whether the product should be produced intracellularly or secreted to the growth medium. Many secretion signals are known. U.S. Patent  
20 No. 4 336 336 describes for procaryotes the use of a leader sequence coding for a non-cytoplasmic protein normally transported to or beyond the cell surface resulting in transfer of the fused protein to the periplasmic space. For yeasts, Kurjan & Herskowitz, Cell  
25 (1982), 30, 933-943 describes a putative  $\alpha$ -factor precursor containing four tandem copies of mature  $\alpha$ -factor, describing the sequence and postulating a processing mechanism. This signal sequence has been used for the secretion of a wide variety of polypeptides from  
30 the yeast *Saccharomyces cerevisiae* ever since the discovery of the signal sequence. Brake et al, PNAS USA, 81, (1984) 4642-4646 gives one example hereof.

Bacteria are not capable of either glycosylating  
35 proteins or, in most cases, forming the correct disulphide bridges in polypeptides of human origin.

Yeast, however, can form correct disulphide bridges, but does not glycosylate proteins in the same manner as higher eucaryotes do. Yeast mutants have been isolated, which glycosylate in a manner similar to mammalian cell lines, thus making yeast a useful host for glycosylated proteins in the future.

The present HBP's are moreover characterized in that they migrate as single bands in SDS-PAGE under reducing conditions, (depicted in Figs. 1 and 2) and have a  $M_r$  of about 28 kDa.

Heparin-binding protein purified from porcine blood platelets has the following amino acid sequence:

```

15  1                                     15
    IleValGlyGlyArgArgAlaGlnProGlnGluPheProPheLeu

                                     30
    AlaSerIleGlnLysGlnGlyArgProPheCysAlaGlyAlaLeu
20

                                     45
    ValHisProArgPheValLeuThrAlaAlaSerCysPheArgGly

                                     60
25  LysAsnSerGlySerAlaSerValValLeuGlyAlaTyrAspLeu

                                     75
    ArgGlnGlnGluGlnSerArgGlnThrPheSerIleArgSerIle

                                     90
30  SerGlnAsnGlyTyrAspProArgGlnAsnLeuAsnAspValLeu

                                     105
    LeuLeuGlnLeuAspArgGluAlaArgLeuThrProSerValAla
35

                                     120
    LeuValProLeuProProGlnAsnAlaThrValGluAlaGlyThr

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12

135  
 AsnCysGlnValAlaGlyTrpGlyThrGlnArgLeuArgArgLeu

5 150  
 PheSerArgPheProArgValLeuArgValThrValThrSerAsn

165  
 ProCysLeuProArgAspMetCysIleGlyValPheSerArgArg

10 180  
 GlyArgIleSerGlnGlyAspArgGlyThrProLeuValCysAsn

195  
 GlyLeuAlaGlnGlyValAlaSerPheLeuArgArgArgPheXxx

196 210  
 XxxSerSerGlyPhePheThrArgValAlaLeuPheArgAsnTrp

20 217  
 IleAspSerValLeuAsnXxx

25 Heparin-binding protein purified from human platelets  
 has the following sequence:

from N-terminal

30 1 15  
 IleValGlyGlyArgLysAlaArgProArgGlnPheProPheLeu

30  
 AlaSerIleGlnAsnGlnGlyArgHisPheCysGlyGlyAlaLeu

35 45  
 IleHisAlaArgPheValMetThrAlaAlaSerCysPheGlnSer

13

60  
 GlnAsnProGlyValSerThrValValLeuGlyAlaTyrAspLeu  
 5 75  
 ArgArgArgGluArgGlnSerArgGlnThrPheSerIleUuuUuu  
 85  
 MetSerGluAsnGlyTyrAspProGlnGln(.....  
 10  
 .....)LeuGlnLeuAspArgGluAlaXxxLeuThrSerXxxVal  
 15 ThrIleLeuProLeuPro(.....)GluAlaGly  
 ThrArgCysGlnValAlaGlyTrpGlySerGlnArg(.....  
 20  
 ..)LeuSerArgPheProArgPheValXxxValThrValThrPro  
 GluAspGlnCysArgProAsnAsnValCysThrGlyValLeuThr  
 25  
 ArgUuuGlyGlyIleCysAsnGlyAspGlyUuuThrProValLeu  
 n-29  
 30 (.....)SerLeuGlyProCys  
 GlyArgGlyProAspPhePheThrArgValAlaLeuPheArgAsp  
 35 n  
 TrpIleAspGlyValLeuAsnAsnProGly

The proteins are furthermore characterized by being glycosylated.

- 5 A computer search of both heparin-binding proteins against a protein data bank using a program from the GENETIC COMPUTER GROUP, University of Wisconsin, shows that the present proteins are heretofore unknown.
- 10 The invention will be explained by the following examples:

Legend to Figures

- 15 Fig. 1 SDS-PAGE under reducing conditions of heparin-binding protein of the porcine type.  
Lane 1: Mr markers  
Lane 2: pHBP
- 20 Fig. 2 SDS-PAGE under reducing conditions (see Example 2) of heparin-binding protein of the human type.  
Lane 1: Mr markers  
Lane 2: hHBP
- 25 Fig. 3 Test for angiogenesis for HBP by using the chick embryo chorioallantoic membrane. For explanation, see Example 5.
- 30 Fig. 4, 5 and 6  
In these pictures HBP treated monocytes (8 ng/ml) (Fig. 4), Endotoxin-treated monocytes (100 ng/ml) (Fig. 5) and PBS-treated monocytes (control cells) (Fig. 6) are shown. For  
35 explanation, see Example 6.



EXAMPLE 1

500 g of blood platelets from porcine blood were suspended in 1.5 l of PBS, frozen and thawed three times by means of liquid nitrogen (N<sub>2</sub>), called Freeze/Thaw below. Freeze/Thaw was centrifuged at 40,000 x g for 30 minutes, and the resulting supernatant was subjected to ultracentrifugation at 300,000 x g for 60 minutes. The resulting supernatant was dialysed for 48 hours against 20 volumes of 10 mM phosphate buffer, 0.5 M NaCl, pH 7.4. The dialysate was pumped on a 5 cm (I.D.) x 10 cm Heparin-Sepharose® Cl-4B column with a flow of 120 ml/h. The column was washed with the same buffer as the sample was dialysed against (buffer A) until no more protein eluted. The column was then eluted with a linear gradient from buffer A to buffer B = 10 mM phosphate buffer, 3 M NaCl, pH 7.4 for 20 hours with a flow of 1.7 ml/min. 200 fractions (during 6 min. each) were collected, and the angiogenic effect was tested. 50 fractions distributed symmetrically around the 1.8 M NaCl fraction in the gradient showed activity. These fractions were pooled and admixed with ove-albumin until a concentration of 0.5 mg/ml. the pooled fractions were dialysed against 20 volumes of buffer A and then pumped on an 0.6 ml Heparin-Sepharose® Cl-4B column with a flow of 6 ml/h. The column was eluted with a linear gradient for 10 hours with a flow of 0.04 ml/min. 120 fractions of 200 µl each were collected and tested for angiogenic activity and then pooled. The pooled fractions were then chromatographed on a reverse phase C<sub>4</sub> column (0.1 ml volume) with a linear gradient from 0 to 80% acetonitrile containing 0.1% trifluoroacetic acid (TFA) for 30 minutes with a flow of 0.025 ml/min. The angiogenic activity was detected in a base line separated top with a retention time of 26 minutes.

SDS-PAGE under reducing conditions shows (see Fig. 1) that the peak contains one component (HBP)  $M_r$  of 28 kDa. The protein in the same peak has the sequence stated in claim 4.

5

## EXAMPLE 2

### Production of HBP from human blood platelets

- 10 100 portions of fresh produced thrombocyte concentrates from healthy blood donors were mixed, and the thrombocytes centrifuged down at 1700 g for 15 min. at 25°C.
- 15 The thrombocytes centrifuged down were suspended in 3 volumes of PBS, and the suspension was frozen and thawed 6 times in liquid N<sub>2</sub>. The suspension was then centrifuged at 40.000 g for 60 min. Supernatants from this were dialyzed for 2 days against 20 volumes of 10
- 20 mM phosphate buffer, 0.5 M NaCl, pH 7.4. The dialysate was pumped on a 5 cm (I.D.) x 10 cm Heparin-Sepharose® Cl-4B column with a flow of 50 ml/h. The column was washed against the same buffer as the sample was dialysed against (buffer A) until no more protein
- 25 eluted. The column was then eluted with a linear gradient from buffer A to buffer B = 10 mM phosphate buffer, 3 M NaCl, pH 7.4 for 20 hours with a flow of 0.90 ml/min. 200 fractions (of 6 min. each) were collected. The fractions were examined by Microbore Reversed Phase C<sub>4</sub>
- 30 column (Aquapore Butyl 100 x 2.1 mm, 7 µm Brownlee Labs) with a gradient as follows:

Time	buffer A 0.1 % TFA	buffer B 70 % CH <sub>3</sub> CN 0.085 % TFA	Flow
5			
0-5 min.	60 %	40 %	200 ul/min.
0-40 min.	30 %	70 %	200 ul/min.

- 10 The Apparatus was an Applied Biosystems 130 A Analyzer, and the protein was monitored at 214 nm. Peaks with a retention time of 20 min. were collected. After drying, the samples were diluted in 0.1 M Tris-Cl, 1 mM EDTA 2.5 % SDS, 0.01 % bromophenyl blue, 5 % 2-mercaptoethanol pH
- 15 8.0. After 5 minutes' at 95°C, the samples were subjected to SDS PAGE by means of Pharmacia Phast Gel equipment with SDS Phast Gel (8-25 %) gels and SDS buffer strips.
- 20 The samples were run at 250 V at 10 mA at 15°C in 60 Vh. (13). The fractions from 70-120 showed a band with M<sub>r</sub>28,000. These fractions were pooled and dialysed with 20 volumes of buffer A. The dialysate was pumped on 1 ml Heparin-Sepharose® Cl-4B column and the column was
- 25 eluted with a linear gradient from buffer A to buffer B for 10 hours with a flow of 0.04 ml/min. Fractions of 200 ul (120) were collected.

### EXAMPLE 3

30

Production of HBP from human material.

- For this purpose, a human cell line, K562, is used. This cell line, which originates from a chronic myeloid
- 35 leucemia patient can be caused to differentiate in megakaryoblastic direction, which is a precursor of the circulating blood platelets, with 12-0-tetradecanoyl

phorbol-14-acetate (TPA). Alitalo et al. (12) has shown that the gene for PDGF is induced in these cells when they are treated with TPA.

5 K562 cells were cultivated in 175 cm<sup>3</sup> Nuclone bottles in RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics. The cell density was adjusted to 300,000 cells per ml, and TPA (Sigma) dissolved in DMSO was added until a concentration of 3 nM. After 3 days,  
10 the cells were sedimented by centrifugation at 500 x g and then washed 1 time in 10 volumes of PBS followed by resedimentation.

10 g of cells harvested in this manner were admixed with  
15 3 volumes of PBS, and the suspension was frozen and thawed 6 times in liquid N<sub>2</sub>. The suspension was ultracentrifuged at 300,000 x g for 60 minutes, and the supernatant was then diluted at 300 ml with 10 mM Na-phosphate buffer pH 7.4 containing 0.5 M NaCl. The  
20 diluted sample was pumped on an 0.5 ml Heparin-Sepharose® Cl-6B within 72 hours. The column was then eluted with a linear gradient for 10 hours from the application buffer (buffer A) to buffer B = 10 mM Na-phosphate buffer pH 7.4 containing 3 M NaCl, and  
25 fractions of 200 µl each were collected.

4 fractions, symmetrical around 1.8 M NaCl, were separately chromatographed on Microbore Reversed Phase C<sub>4</sub> column (Aquapore Butyl 30 x 2.1 mm, 7 µm, Brownlee  
30 Labs) with a linear gradient from 0 to 80% acetonitrile for 30 minutes admixed with 0.1% trifluoroacetic acid (TFA) and a flow of 25 µl/min.

A protein peak with a retention time of 26 minutes is  
35 identical to the retention time for the porcine HBP.

EXAMPLE 4

Detection of angiogenic properties for HBP.

- 5 Male rats of the Wistar family CRL:(W1)BR having a weight of about 240 g and being 80 days old were used. The rats were acclimatized 6 days before use at  $21 \pm 1^\circ\text{C}$ ,  $60 \pm 10\%$  relative humidity with air change 10 times per hour and light from 6.30 a.m. to 6.30 p.m. The rats  
10 were kept in plastic cages with sawdust in the bottom. They were fed ad libitum with Altromin diet 1324 and had free access to drinking water.

- The rats were anaesthetized with pentobarbital 50 mg/kg  
15 body weight by intraperitoneal injection. The rats were shaved on the back and disinfected. Through a 3 cm dorsal cut, the left kidney was exposed, and HBP, absorbed in advance in 10  $\mu\text{l}$  of Affi/Gel® Blue 100-200 mash (wet) 75-150  $\mu$  Bio-Rad dried on a 3 x 3 mm Gelfilm™  
20 absorbable gelatine film Upjohn, was laid below the fibrous capsule of the kidney by a small incision. The surface wound was closed by 5 silk sutures, and Temgesic® was given postoperatively, 0.1 ml dosed twice daily for three days. 5 days postoperatively, the rats  
25 were anaesthetized again with pentobarbital, and the left kidney was exposed. The region around the implant showed clear new vessel formation by macroscopic evaluation.

30 EXAMPLE 5

Test for angiogenesis for porcine and human HBP by using the chick embryo chorioallantoic membrane.

- 35 Fertilized chick eggs on the first day of gestation were placed in a humidified,  $37^\circ\text{C}$  incubator. At day 7 a hole was made in the blunt end of the egg using a 25GS/8 0.5

x 16 needle, and incubation continued. At day 9 a 1 cm x 1 cm "window" was cut through the shell in the pointed end of the eggs and the windows were covered with Tegaderm®. Incubation was continued and at day 11 HBP (5-30ng), absorbed in advance in 3 µl of Affi-Gel® Blue 100-200 mesh (wet) 75-150 µ Bio-Rad dried under laminar airflow on a 3 x 3 mm sterile Gelfile® absorbable gelatine film Upjohn, was laid on the choriollantoic membrane with the affigel towards the membrane and the window was closed again using Tegaderm. After 5 days of incubation at 37°C the responses were microscopically assessed.

Both 30 and 5 ng HBP induced a strong increase in the density of the microvascular bed in the region of the affigel, with apparent regression of larger vessels and typical capillary-ball formations (see drawing).

#### EXAMPLE 6

20

#### Activation of human monocytes

Monocytes were isolated from "buffy coats" of citrated blood from healthy blood donors.

25

Mononuclear cells were isolated as follows: "buffy coats" were diluted with 1 volume of cold RPMI 1640 and layered on the top of 15 ml of Ficoll-Paque in 50 ml Falcon tubes. After centrifugation at 400 x g for 30 minutes in a swingout rotor the layer between Ficoll-Paque and RPMI 1640-plasma (containing mononuclear cells and blood platelets) was removed, and the platelets were removed by washing 3 times (10 min.) at 100 x g.

35

The mononuclear cells were fractionated on a Percoll gradient: 10 x MEM and RPMI 1640 were added to a stock solution of Percoll (density: 1.13 g/ml) to make it isotonical with a density of 1.066 g/ml. A Percoll  
5 gradient was preformed by centrifugation at 3000 x g for 15 min. In a Hereaus medifuge with a 35° fixed angle rotor. On top of this gradient the mononuclear cells were layered and centrifugated at 2.700 x g for 20 minutes in a swingout rotor. In the upper band the  
10 monocytes were found with a purity of more than 90% as determined by non-specific esterase staining. The monocytes were cultivated at a density of  $1 \times 10^6$  cells/ml in 24 well macrowell dishes in RPMI 1640 containing penicillin/Streptomycin. The medium  
15 contained less than 6 pg/ml of endotoxin.

MRC-5 human lung embryo fibroblasts were cultivated in 96 well microwell plates in MEM with 2% FCS at a starting density of  $1 \times 10^4$  cells/ml for 4 days before  
20 testing for mitogenic activity in monocyte culture meddium. The mitogenic activity in 100 µl of monocyte culture medium was determined by pulselabeling the MRX-5 cells with  $^3\text{H}$ -thymidine (1 µCi/ml) from 24 to 42 hours after addition of monocyte medium.

25

#### Results:

When monocytes are incubated with 5 ng HBP, a morphological change is observed after 1 and 2 days of  
30 incubation. The monocytes have become elongated (fig. 4) in a similar manner to monocytes incubated with 100 ng/ml endotoxinin containing 1 mg/ml BSA (bovine serum albumin) (fig. 5). The control cells do not have an activated morphology, as most cells still are uniformly  
35 round shaped (fig. 6). The morphological changes of the monocytes is most clearly observed, when HBP is spotted and dried on the bottom of the well before the monocytes

are added. This may indicate that immobilized HBP is superior to activate monocytes compared to non-immobilized HBP.

- 5 When culture medium from monocytes is tested for mitogenic activity towards MRC-5 human fibroblasts, monocytes incubated with HBP as described above for 2 days are found to secrete about twice the amount of mitogenic activity as control monocytes do. Monocytes  
10 incubated with 100 ng/ml LPS and 21 mg/ml BSA show about 5 times the mitogenic activity as control monocytes do.

#### EXAMPLE 7

#### 15 Topical HBP formulation

HBP was formulated for topical administration in the following composition:

20	Ingredients	% w/w
	distilled water	92.98
	hydroxyethylcellulose	4.0
	sodium chloride	0.41
25	di-sodiumhydrogenphosphate-2hydrate	0.83
	potassiumdihydrogenphosphate	0.28
	gelatine, hydrolyzed	0.5
	benzyl alcohol	1.0
30	to 10 g of the above mentioned composition is added 250 ng HBP.	

#### EXAMPLE 8

- 35 Injectable compositions which are suitable for parenteral administration of HBP contain stabilizers, salts, buffers, preservatives and mixtures thereof. A



simple composition which stabilizes HBP sufficiently for its biological activity to be retained can be injected subcutaneously, intramuscularly or intravenously.

## 5                      Injectable composition

A formulation for parental administration of HBP was prepared with the following composition:

10	Ingradients	% w/v
	glycine	0.15
	de-sodiumhydrogenephosphate	0.026
	sodiumdehydrogenephosphate	0.026
	mannitol	0.74
15	distilled water	100

The formulation may also contain 0.9% (v/v) benzyl alcohol is preservative. To 1 ml of the abovementioned composition is added 25 ng of HBP.

## 20

### EXAMPLE 9

Effect of porcine HBP on wound healing in rats after local administration in wound chambers.

## 25

## SUMMARY

In a wound healing experiment, four groups of twentyfive rats were equipped with wound chambers after removal of the skin on the nape of the neck down to the muscular fascia, in an area of about 15 mm in diameter. The groups were dosed locally with heparin-binding protein (HBP) 12.5 ng, 2.5 ng, 0.5 ng or placebo, twice daily for eight days. A solution of 0.9% saline with 0.1% rat albumin was used as a placebo and as a dissolution medium, and all doses were administered in 100  $\mu$ l volumes. At the two highest dose levels, HBP had a

significant accelerating effect on the wound healing, judged on the basis of the degree of epithelialization. The wounds in the top dose group were richly vascularized.

5

## MATERIALS AND METHODS

### Experimental animals

- 10 100 female Wistar rats, strain CRL:(W1)BR, approximately 240 g b.wt. and 80 days old were used. The rats were purchased from Charles River, BRD, and had been acclimatized for 6 days before use at  $21 \pm 1^\circ\text{C}$ ,  $60 \pm 10\%$  relative humidity, air change 10 times per hour and
- 15 daylight from 06.30 until 18.30 h. The rats were housed singly in rectangular Orth plastic cages with pine bedding. They were fed ad libitum Altromin diet 1324 and had free access to drinking water.

### 20 Operation

- The rats were anaesthetized with pentobarbital, 50 mg/kg b.wt. intraperitoneally. They were shaved on the nape of the neck, and the operating field, 50 mm in diameter,
- 25 was washed, disinfected and stripped with adhesive substance to remove small particles and hairs. Wound chambers consisting of an inner plastic ring and a nylon mesh in adhesive substance were pasted on the skin. The inner diameter of the wound chambers was 16 mm and the
- 30 total diameter 45 mm. The nylon mesh was further fixated to the skin by 12 silk sutures, and the skin within the inner plastic ring was removed down to the muscular fascia. The wounds were covered with polyurethane lids pasted to the chambers with zinc plaster.

35

Postoperatively the rats were given Temgesic®, 0.1 ml doses twice daily for three days.

Dosing

pHBP was administered twice daily in 100 µl volumes of 0.9% NaCl solution with 0.1% rat albumin (Sigma A4538).

5 The dissolution medium was used as placebo.

Postoperatively the rats were randomized into four groups of 25 rats which were dosed as follows, once on the day of operation (day 1) and twice on day 2-8:

10

Group I: 12.5 ng pHBP

Group II: 0.5 ng pHBP

Group III: 2.5 ng pHBP

Group IV: Placebo

15

The doses were administered locally just underneath the wound chamber lids. The cannulae were inserted through the polyurethane lids.

20 Observations

The rats were weighed on days 1, 3, 5, 7 and 9. On day 9 the wound chambers were removed during pentobarbital anaesthesia. The wounds were assessed macroscopically and photographed. The area of and around the operation site was dissected out and fixed in phosphate buffered neutral 4% formaldehyde for later histological examination. Finally, the rats were sacrificed by bleeding from the abdominal aorta. The blood was sampled for serum for IGF-I, PIIINP and hyaluronic acid analyses.

30

RESULTS

35 Table I shows the group mean body weights for the four groups of rats. All the rats lost weight post-operatively, the lowest weights being recorded on day 5.

No significant intergroup differences in body weight were found.

As a result of the macroscopic examination of the wounds, the areas of the wounds and the areas covered with new epithelium on day 9 were calculated in the following way: The two diameters were measured, cranially-caudally and left-right, and the mean diameter ( $D_{total}$ ) was used for calculating the total wound area:

10

$$\left( \frac{D_{total}}{2} \right)^2 \times 3.14 = \text{total area.}$$

15 The newly formed epithelium was measured from the edges, in the two places where it was widest and narrowest, respectively. The two results were added and subtracted from the  $D_{total}$ , giving the  $D_{open}$  for the open wound.

20 The area for the open wound was calculated:

$$\left( \frac{D_{open}}{2} \right)^2 \times 3.14$$

25

and subtracted from the total area giving the area covered with new epithelium. This area was furthermore calculated as per cent of total area. In three rats dosed with 12.5 ng x 2 HBP, the unusual formation of epithelialized peninsulas in the open wound area were observed.

30

The results of the measurements and the calculated areas are shown in the enclosed data sheets. A survey of the results is given in Table II.

35

In many of the rats dosed with the highest dose of HBP (12.5 ng), a red haemorrhagic zone was observed just inside the epithelium edge and on the whole, the wounds seemed richly vascularized. A significantly higher degree of epithelialization was observed for this dose group and for the intermediate dose group (2.5 ng). The total wound area for the various groups was not significantly different from the area in the placebo group.

10

As the fibrin covering the wounds was removed with most of the wound chamber lids, lids + fibrin were fixed together with the tissues for histology.

15 CONCLUSION

On the basis of the macroscopic examination of the wounds, it can be concluded that HBP, 12.5 and 2.5 ng, administered twice daily, accelerated the wound healing significantly, as judged from the degree of epithelialization. The high degree of vascularization of the wounds in the top dose group is due to the angiogenic effect of HBP.

25

30

35

WOUND HEALING IN RATSTable I

5

Group mean body weights in female rats with wound chambers dosed with pBHP or placebo. The rats were operated day 1.

10

	Preparation and dose	No. rats	Body weight, g. means $\pm$ S.E.M.				
			Day 1	Day 3	Day 5	Day 7	Day 9
15	pBHP, 12.5 ng twice daily	23	249 $\pm 2$	240 $\pm 3$	233 $\pm 3$	238 $\pm 3$	242 $\pm 3$
	pBHP, 0.5 ng twice daily	24	245 $\pm 2$	238 $\pm 2$	228 $\pm 2$	234 $\pm 2$	239 $\pm 3$
20	pBHP, 2.5 ng twice daily	24	245 $\pm 2$	243 $\pm 3$	232 $\pm 3$	237 $\pm 3$	240 $\pm 3$
	Placebo (0.9% saline with 0.1% rat albumin	23	245 $\pm 2$	240 $\pm 2$	228 $\pm 2$	233 $\pm 2$	241 $\pm 2$

30

35

WOUND HEALING IN RATSTable II

5                      Macroscopic data obtained with pHBP

Experiment	Placebo	Dose of pHBP administer- ed twice daily	Total area (mm <sup>2</sup> ) means ±S.E.M.	Epithelialized wound area	
				mm <sup>2</sup> means ±S.E.M.	% of total area means ±S.E.M.
15	VII	12.5 ng	118.3 6.3	52.2* 4.1	46.3** 3.6
		2.5 ng	144.3 7.8	53.3* 4.2	39.6 3.0
		0.5 ng	130.2 7.4	44.5 3.8	35.8 3.1
		- (Placebo)	125.1 6.2	38.5 4.2	31.6 3.4
20	albumin				
25					
30					
35					

WOUND HEALING VII			Group I	
	Wound Area <sub>2</sub> (total) mm <sup>2</sup>	Open Wound Area mm <sup>2</sup>	Epithelialized Wound Area mm <sup>2</sup>	% of total area
1	132.73	92.10	40.63	30.61
2	165.13	103.87	61.26	37.10
3	143.14	61.88	81.26	56.77
4	153.94	50.27	103.67	67.35
5	86.59	33.18	53.41	61.68
6	165.13	122.72	42.41	25.68
7	Dead			
8	153.94	86.59	67.35	43.75
9	95.03	50.27	44.76	47.10
10	95.03	44.18	50.85	53.51
11	113.10	70.88	42.22	37.33
12	78.54	50.27	28.27	35.99
13	143.14	122.72	20.42	14.27
14	78.54	28.27	50.27	64.01
15	132.73	103.87	28.86	21.74
16	122.72	95.03	27.69	22.56
17	122.72	63.62	59.10	48.16
18	95.03	28.27	66.76	70.25
19	103.87	40.18	63.69	61.32
20	56.75	12.57	44.18	77.85
21	143.14	103.87	39.27	27.44
22	95.03	50.27	44.76	47.10
23	Dead			
24	113.10	50.27	62.83	55.55
25	132.73	56.75	75.98	57.24
X	118.34		52.17*	46.24**
S.E.M.	6.26		4.05	3.63



	WOUND HEALING VII		Group II	
	Wound Area <sub>2</sub> (total) mm <sup>2</sup>	Open Wound Area mm <sup>2</sup>	Epithelialized Wound Area mm <sup>2</sup>	% of total area
26	201.06	165.13	35.93	17.87
27	132.73	86.59	46.14	34.76
28	113.10	86.59	26.51	23.44
29	188.69	113.10	75.59	40.06
30	132.73	95.03	37.70	28.40
31	103.87	70.88	32.99	31.76
32	143.14	70.88	72.26	50.48
33	103.87	44.18	59.69	57.47
34	122.72	50.27	72.45	59.04
35	50.27	28.27	22.00	43.76
36	132.73	65.75	75.98	57.24
37	103.87	78.54	25.33	24.39
38	95.03	56.75	38.28	40.28
39	95.03	63.62	31.41	33.05
40	86.59	28.27	58.32	67.35
41	153.94	113.10	40.84	26.53
42	113.10	70.88	42.22	37.33
43	176.71	153.94	22.77	12.89
44	103.87	86.59	17.28	16.64
45	143.14	78.54	64.60	45.13
46	143.14	95.03	48.11	33.61
47	143.14	103.87	39.27	27.44
48	Dead			
49	153.94	95.03	58.91	38.27
50	188.69	165.13	23.56	12.49
X	130.21		44.51	35.82
S.E.M.	7.37		3.79	3.06

	WOUND HEALING VII		Group III	
	Wound Area <sub>2</sub> (total) mm <sup>2</sup>	Open Wound Area mm <sup>2</sup>	Epithelialized Wound Area mm <sup>2</sup>	% of total area
51	132.73	95.03	37.70	28.40
52	63.62	44.18	19.44	30.56
53	201.06	122.72	78.34	38.96
54	132.73	56.75	75.98	57.24
55	103.87	44.18	59.69	57.47
56	153.94	70.88	63.06	53.96
57	165.13	70.88	94.75	57.08
58	153.94	78.54	75.40	48.98
59	132.73	78.54	54.19	40.83
60	86.59	38.48	48.11	55.56
61	113.10	50.27	62.83	55.55
62	226.98	188.69	38.29	16.87
63	122.72	78.54	44.18	36.00
64	132.73	113.10	19.63	14.79
65	122.72	70.88	51.84	42.24
66	176.71	143.14	33.57	19.00
67	153.94	78.54	75.40	48.98
68	153.94	95.03	58.91	38.27
69	201.06	132.73	68.33	33.98
70	113.10	63.62	49.48	43.75
71	176.71	153.94	22.77	12.89
72	176.71	132.73	43.98	24.98
73	113.10	78.54	34.56	30.56
74	Dead			
75	153.94	103.87	50.07	32.53
X	144.33		53.33*	39.56
S.E.M.	7.75		4.20	3.03

WOUND HEALING VII			Group IV	
	Wound Area <sub>2</sub> (total) mm <sup>2</sup>	Open Wound Area mm <sup>2</sup>	Epithelialized Wound Area mm <sup>2</sup>	% of total area
76 Dead				
77	103.87	70.88	32.99	31.76
78	95.03	56.75	38.28	40.28
79	95.03	38.48	56.55	59.51
80	70.88	50.27	70.61	29.08
81	132.73	56.75	75.98	57.24
82	86.59	63.62	22.97	26.53
83	113.10	95.03	18.07	15.98
84	176.71	153.94	22.77	12.89
85	113.10	78.54	34.56	30.56
86 Dead				
87	113.10	63.62	49.48	43.75
88	122.72	44.18	78.54	64.00
89	132.72	63.52	69.11	52.07
90	103.87	70.88	32.99	31.76
91	153.94	132.73	21.21	13.78
92	153.94	113.10	40.84	26.53
93	153.94	132.73	21.21	13.78
94	113.10	95.03	18.07	15.98
95	176.71	153.94	22.77	12.89
96	113.10	95.03	18.07	15.98
97	153.94	78.54	75.40	48.98
98	132.73	103.87	28.86	21.74
99	176.71	132.73	43.98	24.89
100	113.10	70.88	42.22	37.33
X	126.12		38.50	31.62
S.E.M.	6.20		4.19	3.37

## WOUND EXPERIMENT - MACROSCOPIC EVALUATION

Rat No.	Wound diameter (mm)		$\bar{x}$	Epithelium edge (mm)		Open wound, diameter (mm)
	1	2		1	2	
1	14	12	13	0.5	0.5	12 (-7x3 mm)
2	15	14	14.5	2	1	11.5
3	15	12	13.5	3	1	9.5 (-3x3 mm)
4	14	14	14	5	1	8
5	11	10	10.5	3	1	6.5
6	15	14	14.5	2	0	12.5
7	Dead					
8	14	14	14	3	0.5	10.5
9	11	11	11	2	1	8
10	13	9	11	3	0.5	7.5
11	14	10	12	2	0.5	9.5
12	10	10	10	1.5	0.5	8
13	13	14	13.5	0.5	0.5	12.5
14	10	10	10	3	1	6
15	13	13	13	1	0.5	11.5
16	12	13	12.5	1	0.5	11
17	13	12	12.5	3	0.5	9
18	12	10	11	4	1	6
19	12	11	11.5	2	2	7.5 (-2x2 mm)
20	8	9	8.5	4	0.5	4
21	13	14	13.5	1	1	11.5
22	11	11	11	2	1	8
23	Dead					
24	12	12	12	4	0	8
25	13	13	13	3	1.5	8.5

## WOUND EXPERIMENT - MACROSCOPIC EVALUATION

Rat No.	Wound diameter (mm)		$\bar{x}$	Epithelium edge (mm)		Open wound, diameter (mm)
	1	2		1	2	
26	16	16	16	1	0.5	14.5
27	12	14	13	1.5	1	10.5
28	12	12	12	1	0.5	10.5
29	16	15	15.5	3	0.5	12.
30	13	13	13	1	1	11
31	12	12	11.5	1.5	0.5	9.5
32	14	13	13.5	2	2	9.5
33	11	12	11.5	2	2	7.5
34	14	11	12.5	4	0.5	8
35	8	8	8	1.5	0.5	6
36	13	13	13	2.5	2	8.5
37	13	10	11.5	1	0.5	10
38	11	11	11	2	0.5	8.5
39	10	12	11	1.5	0.5	9
40	12	9	10.5	2	2.5	6
41	14	14	14	1.5	0.5	12
42	12	12	12	2.5	0.5	9.5
43	15	15	15	0.5	0.5	14
44	11	12	11.5	1	0	10.5
45	15	12	13.5	2.5	1	10
46	14	13	13.5	2	0.5	11
47	14	13	13.5	2	0	11.5
48 Dead						
49	15	13	14	3	0	11
50	16	15	15.5	0.5	0.5	14.5

## WOUND EXPERIMENT - MACROSCOPIC EVALUATION

Rat No.	Wound diameter (mm)		$\bar{X}$	Epithelium edge (mm)		Open wound, diameter (mm)
	1	2		1	2	
51	14	12	13	1	1	11
52	9	9	9	1	0.5	7.5
53	16	16	16	3	1	8.5
54	14	12	13	3.5	1	8.5
55	12	11	11.5	2.5	1.5	7.5
56	14	14	14	3.5	1	9.5
57	15	14	14.5	4	1	9.5
58	15	13	14	3.5	0.5	10
59	13	13	13	3	0	10
60	11	10	10.5	3	0.5	7
61	12	12	12	4	0	8
62	17	17	17	1	0.5	15.5
63	13	12	12.5	2	0.5	10
64	12	14	13	1	0	12
65	14	11	12.5	3	0	9.5
66	15	15	15	1	0.5	13.5
67	14	14	14	2	2	10
68	13	15	14	2	1	11
69	16	16	15	2	1	13
70	12	12	12	2	1	9
71	15	15	15	0.5	0.5	14
72	16	14	15	1.5	0.5	13
73	12	12	12	-	-	10
74 Dead						
75	15	13	14	2	0.5	11.5

## WOUND EXPERIMENT - MACROSCOPIC EVALUATION

Rat No.	Wound diameter (mm)		X		Epithelium edge (mm)		Open wound, diameter (mm)
	1	2			1	2	
76 Dead							
77	11	12	11.5	2	0		9.5
78	11	11	11	2	0.5		8.5
79	10	12	11	2	2		7
80	10	9	9.5	1.5	0		8
81	13	13	13	3	1.5		8.5
82	12	9	10.5	1	0.5		9
83	13	11	12	1	0		11
84	15	15	15	0.5	0.5		14
85	12	12	12	2	0		10
86 Dead							
87	13	11	12	2	1		9
88	12	13	12.5	3	2		7.5
89	13	13	13	3	1		9
90	12	11	11.5	2	0		9.5
91	14	14	14	1	0		13
92	14	14	14	2	0		12
93	14	14	14	0.5	0.5		13
94	14	10	12	1	0		11
95	14	16	15	0.5	0.5		14
96	13	13	13	1	1		11
97	14	14	14	3	1		10
98	13	13	13	1	0.5		11.5
99	14	16	15	1.5	0.5		13
100	11	13	12	2	0.5		9.5

### Histological evaluation

#### Materials:

5

A 5 µm thick slice is cut out in the centre (cranio-caudal) from the paraffin embedded wound.

10 The slice is stained in hematoxylin-cosin and evaluated in light microscope.

#### Results:

15 As a result of the microscopic examination of the wounds, the new epithelium on day 9 was calculated in the following way:

The diameter of the total wound was measured and the diameter of the open wound was measured and subtracted:

20

$$\frac{\text{Total wound} - \text{Open wound}}{2} = \text{new epithelium}$$

25 A rating scale was used to combine a quantitative and a qualitative histological evaluation of the wound healing in rats:

30

35



$$\begin{array}{c}
 \text{new} \\
 \text{epithe-} \\
 \text{lium} \\
 \text{(mm)}
 \end{array}
 + \left( \begin{array}{c} \text{evaluation} \\ \text{of granu-} \\ \text{lation} \\ \text{tissue} \\ \text{(0--4)} \end{array} \right)
 + \left( \begin{array}{c} \text{giant} \\ \text{cells} \\ \text{(0--4)} \end{array} \right)
 + \left( \begin{array}{c} \text{height of} \\ \text{granula-} \\ \text{tion} \\ \text{tissue} \\ \text{(mm)} \end{array} \right)
 + \left( \begin{array}{c} \text{evaluation} \\ \text{of epithe-} \\ \text{lium (0--4)} \end{array} \right)$$


---

4

= rating

The areas of the wounds and the areas covered with new epithelium on day 9 were calculated in the following way:

$$\text{Total area} = \left( \frac{\text{total wound diameter}}{2} \right)^2 \times 3.14$$

New epithelium area =

$$\text{Total area} = \left( \frac{\text{open wound diameter}}{2} \right)^2 \times 3.14$$

and the new epithelium area was furthermore calculated as per cent of total area.

The results of the measurements and the calculated ratings and areas are as shown in the Tables.

### CONCLUSION

On the basis of the microscopic examination of the wounds and the evaluation of the data in the rating scale, it may be concluded that p-HBP, doses 12.5 ng and 0.5 ng, has an effect on the wound healing.

The effect is seen on the granulation tissues which are high in group 1 and 2, doses 12.5 ng and 0.5 ng pHBP

(table II). In group 1, dose 12.5 ng pHBP, the granulation tissues are more mature compared to the placebo group (table II). From the area calculation the degree of epithelialization shows no significant difference between the groups.

## WOUND HEALING EXPERIMENT VII

Table 1

10 Microscopic data obtained with pHBP

15	Group	Rating scale	
		Mean $\pm$ S.E.M.	
20	1		
	12.5 ng	2.53 ***	
	p-HBP	0.06	
25	2		
	0,5 ng	2.35 *	
	p-HBP	0.07	
30	3		
	2.5 ng	2.26	
	p-HBP	0.07	
35	4		
	Placebo	2.10	
		0.09	

\*\*\* p < 0.001 levels of significant difference from placebo

\*\* p < 0.01

\* p < 0.05

## WOUND HEALING EXPERIMENT VII

Table II

Group	Total wound (mm)	Open wound (mm)	Granulation tissue (0-4)	Giant cells (0-4)	Epithelium edge (mm)	Evaluation of epithelium (0-4)	Hight of granulation tissue (mm)
1	X 9.26*	6.52	2.96*	0.48*	2.78	4.00	2.26**
12.5 ng HBP	Sd 1.91 SEM 0.40	2.19 0.46	0.21 0.04	1.08 0.23	1.13 0.23	0.00 0.00	0.54 0.11
2	X 9.33*	6.88*	2.58	0.58*	2.46	4.00	2.17**
0.5 ng p-HBP	Sd 1.86 SEM 0.38	2.33 0.48	0.50 0.10	1.06 0.22	1.22 0.25	0.00 0.00	0.56 0.12
3	X 8.88	6.54	2.63	0.63	2.33	4.00	1.88
2.5 ng p-HBP	Sd 2.11 SEM 0.43	2.06 0.42	0.49 0.10	1.28 0.26	1.13 0.23	0.00 0.00	0.61 0.13
4	X 8.22	5.39	2.70	1.39	2.83	4.00	1.70
Placebo	Sd 1.35 SEM 0.27	1.85 0.39	0.47 0.10	1.50 0.31	1.34 0.28	0.00 0.00	0.56 0.12

\*\*\* p &lt; 0.001 levels of significant difference from placebo

\*\* p &lt; 0.01

\* p &lt; 0.05

P a t e n t   C l a i m s

1. A heparin-binding protein which has, in glycosylated state, an apparent molecular weight of about 28 kDa,  
5 determined by SDS-PAGE under reducing conditions, and exhibits angiogenic properties in vivo.
2. A heparin-binding protein according to claim 1,  
c h a r a c t e r i z e d in that it is glycosylated.  
10
3. A heparin-binding protein according to claim 2 of the porcine type, c h a r a c t e r i z e d in that it is glycosylated at Asn 113.
- 15 4. A heparin-binding protein of the porcine type, c h a r a c t e r i z e d by having the amino acid sequence:

20

25

30

35

1 15  
IleValGlyGlyArgArgAlaGlnProGlnGluPheProPheLeu  
30  
5 AlaSerIleGlnLysGlnGlyArgProPheCysAlaGlyAlaLeu  
45  
ValHisProArgPheValLeuThrAlaAlaSerCysPheArgGly  
10 60  
LysAsnSerGlySerAlaSerValValLeuGlyAlaTyrAspLeu  
75  
ArgGlnGlnGluGlnSerArgGlnThrPheSerIleArgSerIle  
15 90  
SerGlnAsnGlyTyrAspProArgGlnAsnLeuAsnAspValLeu  
105  
20 LeuLeuGlnLeuAspArgGluAlaArgLeuThrProSerValAla  
120  
LeuValProLeuProProGlnAsnAlaThrValGluAlaGlyThr  
25 135  
AsnCysGlnValAlaGlyTrpGlyThrGlnArgLeuArgArgLeu  
150  
PheSerArgPheProArgValLeuArgValThrValThrSerAsn  
30 165  
ProCysLeuProArgAspMetCysIleGlyValPheSerArgArg  
180  
35 GlyArgIleSerGlnGlyAspArgGlyThrProLeuValCysAsn

195

GlyLeuAlaGlnGlyValAlaSerPheLeuArgArgArgPheXxx

196

210

5 XxxSerSerGlyPhePheThrArgValAlaLeuPheArgAsnTrp

217

IleAspSerValLeuAsnXxx

10 wherein X195 and X196 are arbitrary amino acids, and  
X217 is one or two arbitrary amino acids.

5. A heparin-binding protein of the porcine type according to claim 4, c h a r a c t e r i z e d in that  
15 X195 = Asn, X196 = Lys.

6. A heparin-binding protein of the porcine type according to claim 4 and 5, c h a r a c t e r i z e d in that X217 is the two amino acids in the sequence AsnPro.

7. A heparin-binding protein of the human type,  
c h a r a c t e r i z e d by having the following amino  
acid sequences:

from N-terminal

1 15  
IleValGlyGlyArgLysAlaArgProArgGlnPheProPheLeu

30

30  
AlaSerIleGlnAsnGlnGlyArgHisPheCysGlyGlyAlaLeu

35

35 IleHisAlaArgPhe

C-terminal

n-15

n-1 n

ValAlaLeuPheArgAspTrpIleAspGlyValLeuAsnAsnProGly

5 wherein n denotes the total number of amino acids in the protein sequence.

8. A heparin-binding protein according to claim 7,  
c h a r a c t e r i z e d by having the following amino  
10 acid sequence:

from N-terminal

1

15

15 IleValGlyGlyArgLysAlaArgProArgGlnPheProPheLeu

30

AlaSerIleGlnAsnGlnGlyArgHisPheCysGlyGlyAlaLeu

20

45

IleHisAlaArgPheValMetThrAlaAlaSerCysPheGlnSer

60

GlnAsnProGlyValSerThrValValLeuGlyAlaTyrAspLeu

25

73

ArgArgArgGluArgGlnSerArgGlnThrPheSerIle

30 C-terminal

n-29

n-15

SerLeuGlyProCysGlyArgGlyProAspPhePheThrArgVal

35

n

AlaLeuPheArgAspTrpIleAspGlyValLeuAsnAsnProGly

wherein n has the significance defined in claim 7.

9. A heparin-binding protein according to claim 8 also comprising the following amino acid sequences between  
5 the amino acids Gln (69) and Ser (n-29):

from N-terminal

1	15
10	IleValGlyGlyArgLysAlaArgProArgGlnPheProPheLeu
	30
	AlaSerIleGlnAsnGlnGlyArgHisPheCysGlyGlyAlaLeu
15	45
	IleHisAlaArgPheValMetThrAlaAlaSerCysPheGlnSer
	60
	GlnAsnProGlyValSerThrValValLeuGlyAlaTyrAspLeu
20	
	73
	ArgArgArgGluArgGlnSerArgGlnThrPheSerIle
25	69
	GlnThrPheSerIleUuuUuuMetSerGluAsnGlyTyrAspPro
	GlnGln
	-----
30	
	LeuGlnLeuAspArgGluAlaXxxLeuThrSerXxxValThrIle
	LeuProLeuPro
	-----
35	
	GluAlaGlyThrArgCysGlnValAlaGlyTrpGlySerGlnArg
	-----



LeuSerArgPheProArg  
-----

5 PheValXxxValThrValThrProGluAspGlnCysArgProAsn

AsnValCysThrGlyValLeuThrArg  
-----

10 UuuGlyGlyIleCysAsnGlyAspGlyUuuThrProValLeu  
-----

wherein Uuu is an unknown amino acid and Xxx is a possible glycosylation site, probably Asn.

15

C-terminal

20 n-29 n-15  
SerLeuGlyProCysGlyArgGlyProAspPhePheThrArgVal

n  
AlaLeuPheArgAspTrpIleAspGlyValLeuAsnAsnProGly

25

wherein n has the significance defined in claim 7.

10. A heparin-binding protein according to claim 8, having the following structure:

30

from N-terminal

1 15  
IleValGlyGlyArgLysAlaArgProArgGlnPheProPheLeu

35

30  
AlaSerIleGlnAsnGlnGlyArgHisPheCysGlyGlyAlaLeu

48

45  
IleHisAlaArgPheValMetThrAlaAlaSerCysPheGlnSer  
5 60  
GlnAsnProGlyValSerThrValValLeuGlyAlaTyrAspLeu  
75  
ArgArgArgGluArgGlnSerArgGlnThrPheSerIleUuuUuu  
10  
85  
MetSerGluAsnGlyTyrAspProGlnGln(.....  
15 .....)LeuGlnLeuAspArgGluAlaXxxLeuThrSerXxxVal  
ThrIleLeuProLeuPro(.....)GluAlaGly  
20  
ThrArgCysGlnValAlaGlyTrpGlySerGlnArg(.....  
..)LeuSerArgPheProArgPheValXxxValThrValThrPro  
25  
GluAspGlnCysArgProAsnAsnValCysThrGlyValLeuThr  
30 ArgUuuGlyGlyIleCysAsnGlyAspGlyUuuThrProValLeu  
n-29  
(.....)SerLeuGlyProCys  
35  
GlyArgGlyProAspPhePheThrArgValAlaLeuPheArgAsp

n  
TrpIleAspGlyValLeuAsnAsnProGly

wherein n has the significance defined in claim 7; Uuu  
5 and Xxx have the significance defined in claim 9.

11. A therapeutic preparation, c h a r a c t e r i z e d  
in that it contains a therapeutically active amount of  
the heparin-binding protein of porcine type according to  
10 claims 1-6.

12. A therapeutic preparation, c h a r a c t e r i z e d  
in that it contains a therapeutically active amount of  
the heparin-binding protein of the human type according  
15 to claims 7-10.

13. A process for producing the heparin-binding protein  
of porcine type according to claims 1-6, c h a r a c -  
t e r i z e d by extracting porcine thrombocytes, and  
20 purifying the extract by chromatography on heparin-  
Sephrose and Reversed Phase HPLC for isolation of  
heparin-binding protein.

14. A process for producing the heparin-binding protein  
25 of the human type according to claims 7-10,  
c h a r a c t e r i z e d by extracting human thrombo-  
cytes, and purifying the extract by chromatography on  
Heparin-Sephrose and Reversed Phase HPLC for isolation  
of the protein.

30  
15. A process according to any of claims 13 and 14,  
c h a r a c t e r i z e d by performing the application  
of the cell extracts on heparin-Sephrose in 0.5 molar  
NaCl solution adjusted to a pH value of 7.2 to 7.6,  
35 preferably 7.4.

16. A process for producing heparin-binding protein, characterized by using biosynthetic recombinant technique.

5 17. A DNA structure coding for the heparin-binding protein according to any of claims 1-6, 11 and 13.

18. A cDNA coding for the heparin-binding protein of human type according to claims 7-10, 12 and 14.

10

15

20

25

30

35

1/6

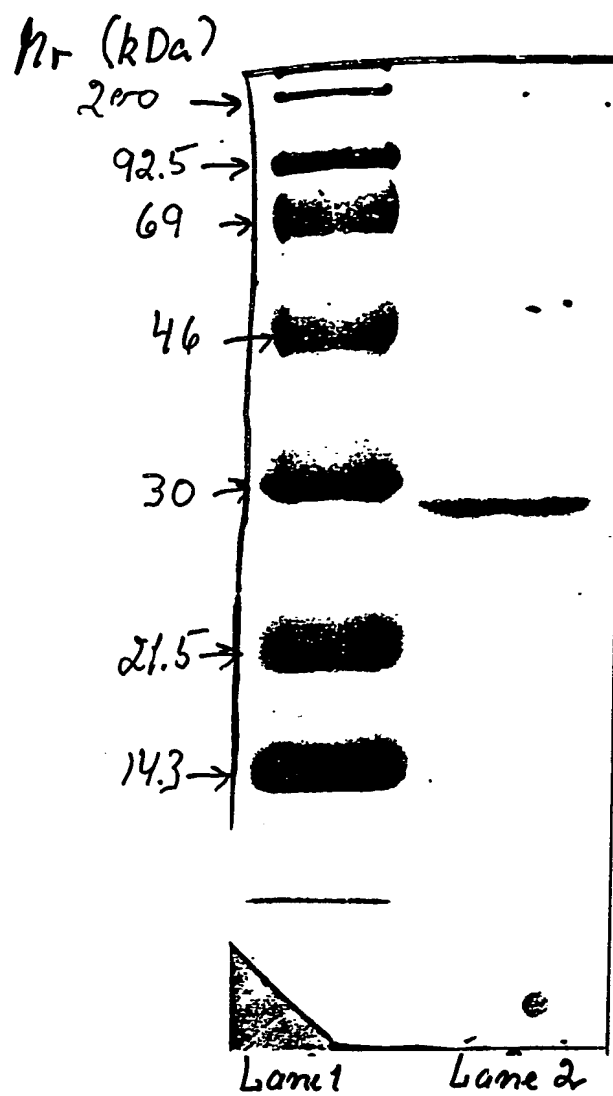


FIG . 1

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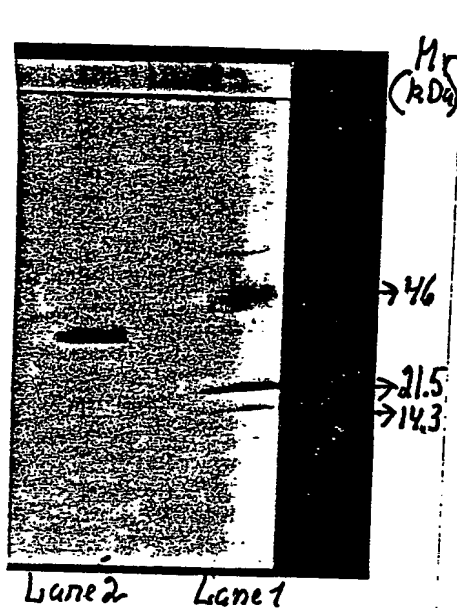


FIG. 2

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FIG. 3

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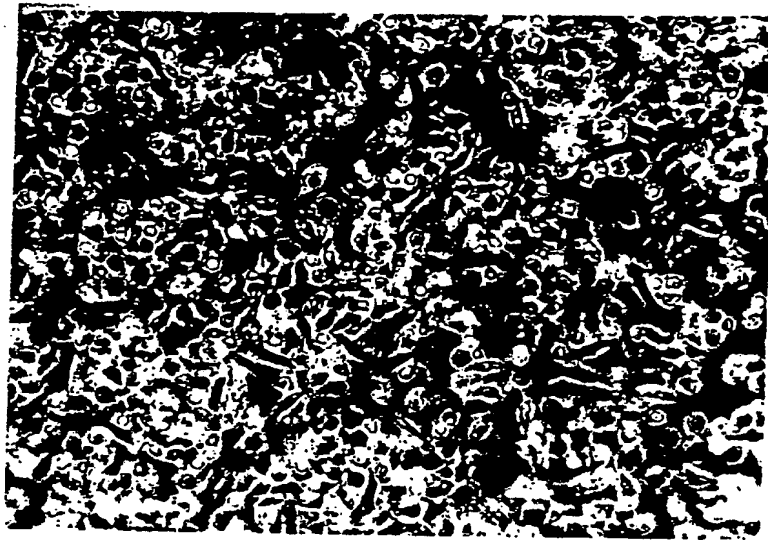


FIG. 4

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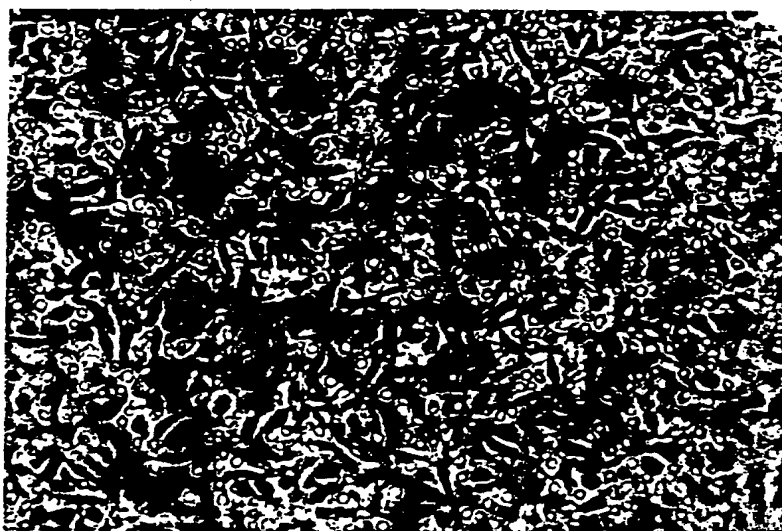


FIG. 5

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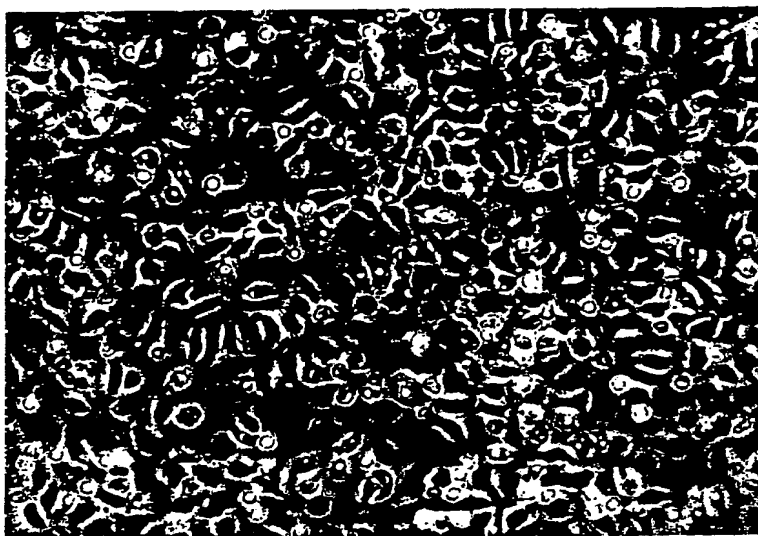


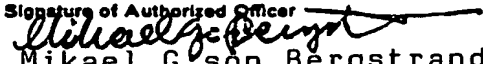
FIG. 6

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# INTERNATIONAL SEARCH REPORT

International Application No PCT/DK89/00059

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) *		
According to International Patent Classification (IPC) or to both National Classification and IPC <sup>4</sup>		
C 07 K 13/00, A 61 K 37/02, C 12 N 15/00		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
IPC 4	C 07 K; C 12 N; A 61 K	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched *		
SE, NO, DK, FI classes as above.		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT</b> <sup>9</sup>		
Category *	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X	Dialog Information Services, File 55, BIOSIS 81-89, BIOSIS Number 85050038, Rauvala H et al: "Isolation and some characteristics of an adhesive factor of brain that enhances neurite outgrowth in central neurons", J Biol Chem 262(34), 1987, 16625-16635	1-3
X	Dialog Information Services, File 55, BIOSIS 81-89, BIOSIS Number 83082612, Pande H et al: Demonstration of struc- tural differences between the two sub- units of human-plasma fibronectin in the carboxyl-terminal heparin-binding domain" Eur J Biochem 162(2), 1987, 403-412	1-3
X	Dialog Information Services, File 55, BIOSIS 81-89, BIOSIS Number 79028069, Raugi G J et al: "Location and partial characterization of the heparin-binding fragment of platelet thrombospondin", Thromb Res 36(2), 1984, 165-176 .../...	1
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents: <sup>10</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p> </div> </div>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
1989-06-16	1989 -06- 26	
International Searching Authority	Signature of Authorized Officer	
Swedish Patent Office	 Mikael G. son Bergstrand	

## III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	WO, A1, 88/10269 (MONASH UNIVERSITY) 29 December 1988 See the whole document.	1
A	WO, A1, 87/07183 (NEW YORK UNIVERSITY) 3 December 1987 See the whole document	1
A	EP, A1, 0 298 723 (BIOTECHNOLOGY RESEARCH ASSOCIATES, J V) 11 January 1989 See the whole document	1
A	EP, A2, 0 241 136 (THE PRESIDENT AND FELLOWS OF HARVARD COLLEGE) 14 October 1987 See the whole document	1